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ORIGINAL PAPER

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Impact of the Mg^{2+}-citrate transporter CitM on heavy metal toxicity in *Bacillus subtilis*

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Abstract *Bacillus subtilis* possesses a secondary transporter, CitM, that is specific for the complex of citrate and Mg^{2+} but is also capable of transporting citrate in complex with the heavy metal ions Zn^{2+}, Ni^{2+} and Co^{2+}. We report on the impact of CitM activity on the toxicity of Zn^{2+}, Ni^{2+} and Co^{2+} in *B. subtilis*. In a *citM* deletion mutant or under conditions in which CitM is not expressed, the toxic effects of the metals were reduced by the presence of citrate in the medium. In contrast, the presence of citrate dramatically enhanced toxicity when the Mg^{2+}-citrate transporter was present in the membrane. It is demonstrated that the complex of Ni^{2+} and citrate is transported into the cell and that the uptake is responsible for the enhanced toxicity. At toxic concentrations of the metal ions, the cultures adapted by developing tolerance against these ions. Tolerant cells isolated by exposure to one of the metal ions remained tolerant after growth in the absence of toxic metal ions and were cross-tolerant against the other two toxic ions. Tolerant strains were shown to contain point mutations in the *citM* gene, which resulted in premature termination of translation.

Keywords *Bacillus subtilis* · Citrate transporter · CitM · Heavy metal · toxicity

Introduction

A limited number of bacteria of the genera *Pseudomonas*, *Citrobacter*, *Bacillus* and *Klebsiella* have been described that transport metal-citrate complexes across the cytoplasmic membrane into the cell (Willecke et al. 1973; Bergsma and Konings 1983; Madsen and Alexander 1985; Brynhildsen and Rosswall 1989; Joshi-Tope and Francis 1995). The Mg^{2+}-citrate transporter CitM of *Bacillus subtilis* is the best-studied example and accepts the toxic heavy metal ions Zn^{2+}, Ni^{2+} and Co^{2+} instead of Mg^{2+} in the metal-citrate complex (Krom et al. 2000). Bacteria capable of taking up and metabolizing heavy metal ions complexed to citrate have been implicated in preventing the mobilization of toxic metals from waste dumps (Joshi-Tope and Francis 1995). Alternatively, these organisms may play a role in bioremediation of heavy-metal-polluted sites by active accumulation of the toxic ions after heterotrophic leaching with citrate (White et al. 1997; Peters 1999).

Transport of citrate across the cytoplasmic membrane is an active process mediated by a designated transporter protein. All known bacterial citrate transporters are secondary transporters that use the energy stored in electrochemical gradients of protons or sodium ions to drive uptake. Most citrate transporters recognize free citrate, but it has long been known that in *B. subtilis* citrate transport is dependent on the presence of divalent cations (Willecke et al. 1973; Bergsma and Konings 1983). Two transporters, members of a distinct transporter family (Boorsma et al. 1996), mediate transport of Me^{2+}-citrate into the cell. The transporters, CitM and CitH, have complementary metal ion specificity; CitM transports complexes of citrate with Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} while CitH transports complexes of citrate with Ca^{2+}, Sr^{2+} and Ba^{2+} (Krom et al. 2000). CitM is the transporter responsible for growth of *B. subtilis* on citrate as the carbon and energy source. Expression of CitM is under strict control of the medium composition (Warner et al. 2000). The transporter is induced by citrate via a two-component system (Yamamoto et al. 2000) and repressed by rapidly metabolized substrates including the sugars glucose, glycerol, and inositol, but also by succinate/glutamate. Disruption of the *ccpA* gene, coding for a central component of the carbon catabolite repression system, was shown to relieve the repression. The physiological function of CitH is not known.

B. subtilis expressing CitM is potentially useful in the removal of Zn^{2+}, Ni^{2+}, and Co^{2+} from waste water, e.g. such as obtained after heterotrophic leaching. A potential

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drawback is increased toxicity of the heavy metal ions when the transporter is active. Moreover, increased toxicity may induce resistance and/or tolerance mechanisms that may compromise the application. In this report, the impact of CitM activity on metal ion toxicity and the development of resistance and/or tolerance in *B. subtilis* when Zn^{2+}, Ni^{2+}, and Co^{2+} are present in the growth medium were evaluated. It is demonstrated that CitM dramatically enhances the toxicity of the metal ions in the presence of citrate, but the cells rapidly adapt to the hostile environment by inactivation of the CitM transporter.

Materials and methods

Bacterial strains, media, and growth conditions

The *B. subtilis* strains 168 (*trpC2*), CM010 (*trpC2 ccpA*::Tn*917 spc amyE*::P*citM-lacZ*; (Warner et al. 2000) and CITMd (*trpC2* Δ*citM ery*; Prof. Junichi Sekiguchi, Shinshu University, Japan) were routinely grown at 37 °C while shaking vigorously in flasks containing Luria Bertani broth (LB) or LB supplemented with 10 mM citrate. When appropriate, erythromycin, chloramphenicol and spectinomycin were added at a final concentration of 0.3 µg/ml, 5 µg/ml and 100 µg/ml, respectively. Metals were added as $MeCl_2$ dissolved in Milli-Q and autoclaved. Citrate was added from a 1 M stock solution of tri-sodium citrate (pH 6.5) dissolved in Milli-Q that was autoclaved separately. Cultures were inoculated with overnight cultures grown in LB at a dilution of 1:100. Zn^{2+}-, Ni^{2+}-, and Co^{2+}-resistant strains (metal-tolerant strains; see below) were isolated as follows. LB medium supplemented with 10 mM citrate and 0.6 mM $ZnCl_2$, 1 mM $NiCl_2$, or 0.4 mM $CoCl_2$ was inoculated with a single colony from an LB plate containing strain CM010. After growth for 24 h, the cells were plated on LB and single colonies were selected for further experiments. Growth was monitored by measuring the optical density at 660 nm (OD_{660}) using a Hitachi U-1100 spectrophotometer. The growth rate was inferred from the exponential part of the growth curve. Experiments were done at least in triplicate and typical experiments are shown.

Uptake in whole cells

[1,5-^{14}C]citrate (114 mCi/mmol) or $^{63}NiCl_2$ (1 mCi/mg) (Amersham Pharmacia Biotech) uptake was measured according to the rapid-filtration method as described previously with minor modifications (Lolkema et al. 1994). Briefly, cells were harvested by centrifugation and washed once with ice-cold 50 mM PIPES (pH 6.5) after which they were resuspended to an OD_{660} of 10. Cells were diluted ten-fold in 50 mM PIPES (pH 6.5) and 100-µl samples were incubated 8 min at 30 °C while stirring magnetically. At time zero, [1,5-^{14}C]citrate or $^{63}NiCl_2$ was added yielding a final concentration of 4.4 µM and 12.5 µM, respectively. Uptake was stopped by the addition of 2 ml ice-cold 0.1 M LiCl solution, immediately followed by filtering through a 0.45-µm pore-size nitrocellulose filter. The filter was washed once with 2 ml ice-cold 0.1 M LiCl, after which the filters were submerged in scintillation fluid and the retained radioactivity was counted in a liquid scintillation counter. The background was estimated by adding the radiolabeled substrate to the cell suspension after the addition of 2 ml ice-cold LiCl, followed by immediate filtering. Experiments were done at least in triplicate and typical experiments are shown.

β-Galactosidase assay

B. subtilis strain CM010 contains the *lacZ* gene fused behind the promoter region of *citM* (P*citM-lacZ*) integrated in the *amyE* locus of a CcpA-deficient strain (Warner et al. 2000). β-Galactosidase activity was measured by harvesting 1 ml of culture by centrifugation for 5 min in an Eppendorf tabletop centrifuge operated at 14,000 rpm. Cell extracts were obtained by lysozyme treatment, and β-galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside as the substrate as described previously (Miller 1972).

Cloning of *citM* from metal-tolerant strains

Escherichia coli strain DH5α (Invitrogen) was used for cloning and sequencing purposes and routinely grown in LB medium containing ampicillin at a concentration of 100 µg/ml. Chromosomal DNA of three metal-tolerant strains was isolated using a standard protocol. A 2.1-kb fragment containing the promoter region of *citM* (P*citM*) and the *citM* gene was amplified by PCR using *Pwo* DNA polymerase for high-fidelity amplification (Roche Molecular Biochemicals, Mannheim Germany). The forward primer (5′-CTCCAAG*GAATTC*CAGACGGTTGCATTGCC-3′) introduced an *Eco*RI site (italic) and the reverse primer (5′-GC*TCTAGA*TCATACGGAAATAGAGATCGCA-3′) introduced a *Xba*I site (italic) downstream of the stop codon (Warner et al. 2000). The PCR fragment of 2.1 kb was digested with *Eco*RI and *Xba*I and ligated into pBluescript SK+ (Stratagene, La Jolla, Calif., USA) digested with the same enzymes. Subsequently, using a unique internal *Nco*I site, two fragments of 0.8 and 1.3 kb were subcloned into pBluescript SK+ and pET302 (van der Does et al. 1998), respectively, and sequenced (Baseclear, Leiden, The Netherlands).

Results

The effect of citrate on metal ion toxicity in *B. subtilis*

B. subtilis 168 was grown in LB containing increasing concentrations of Zn^{2+}, Ni^{2+} and Co^{2+} (Fig. 1). The growth rate started to decrease at a concentration of 0.4 mM $ZnCl_2$ and growth was abolished at 1 mM $ZnCl_2$. The effect of $NiCl_2$ was less dramatic; the growth rate decreased at concentrations higher than 1 mM and no growth was observed above 2.5 mM. $CoCl_2$ was the most toxic, with a severe effect on the growth rate at a concentration as low as 0.6 mM $CoCl_2$. Therefore, the toxicity of metal ions for the growth of *B. subtilis* in LB medium in the absence of citrate decreased in the order Co^{2+}>Zn^{2+}>Ni^{2+}.

Addition of citrate to the growth medium may have two effects: (1) it will chelate divalent metal ions, thereby reducing the concentration of free ions; (2) it may induce expression of the Mg^{2+}-citrate transporter CitM, which introduces an entrance pathway for the Me^{2+}-citrate complex. To distinguish between the two effects, the toxicity of Zn^{2+}, Ni^{2+} and Co^{2+} in the presence of citrate was first investigated using a *citM* deletion strain of *B. subtilis* (strain CITMd, a gift from Prof. Junichi Sekiguchi, Shinshu University, Japan). Citrate protected *B. subtilis* CITMd against the toxic effects of the three metal ions (Fig. 1). Significant growth of strain CITMd was observed at a concentration of 1 mM $ZnCl_2$ when the medium was supplemented with 10 mM citrate, while no growth of the wild-type strain was observed at the same concentration in the absence of citrate. Similarly, while *B. subtilis* 168 ceased to grow in the presence of $NiCl_2$ and $CoCl_2$ concentrations of 2.5 and 1 mM, respectively, significant growth of strain CITMd was observed in the presence of citrate.

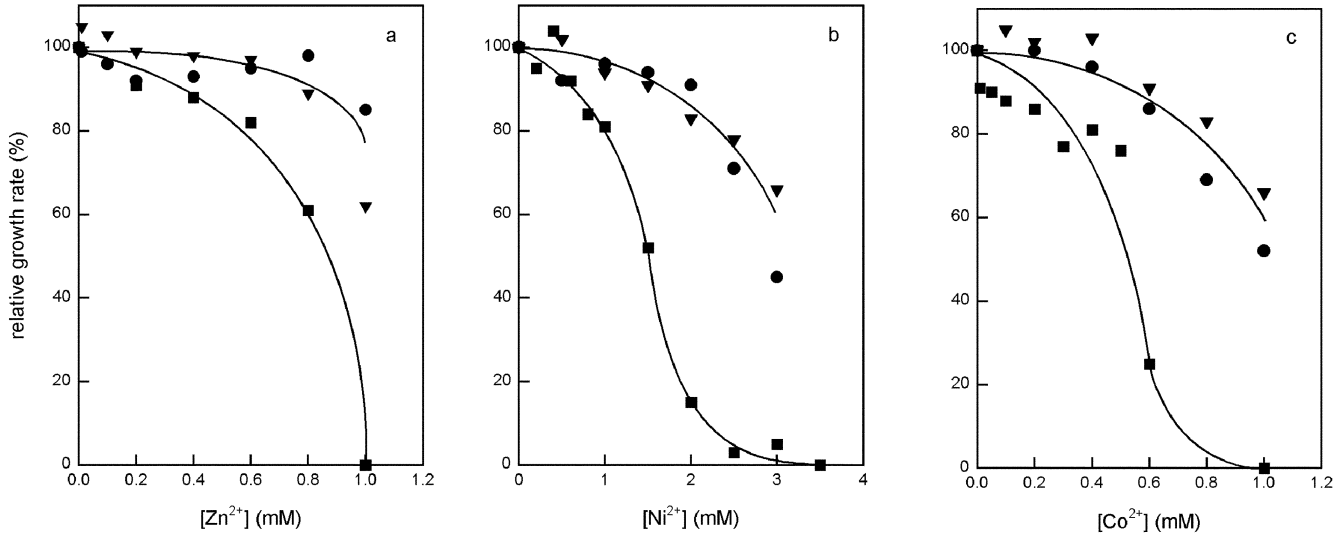

Fig. 1 Growth rates of *B. subtilis* wt (●, ■) and a *citM* deletion strain (▼) in LB medium containing Zn^{2+} (**a**), Ni^{2+} (**b**) and Co^{2+} (**c**) in the presence (●, ▼) and absence (■) of 10 mM citrate. Growth rates are indicated as the percentage of the growth rate in the absence of added metal ions in the medium (1.2–1.5 h^{-1})

At higher metal concentrations, the growth rate of CITMd in the presence of citrate was affected for all metals tested, most likely because the citrate concentration was no longer sufficient to chelate all of the metal ions.

The toxicity of Zn^{2+}, Ni^{2+} and Co^{2+} in the presence of citrate was not significantly different for wild-type *B. subtilis* and the *citM* deletion strain (Fig. 1). Control experiments demonstrated that CitM was not expressed when cells were grown in LB medium supplemented with citrate. The activity of CitM is routinely measured by the uptake of ^{14}C-citrate in the presence of Ni^{2+} under conditions in which all citrate is present as the Ni^{2+}-citrate complex (Krom et al. 2000; Warner et al. 2000). The *citM* deletion strain grown in minimal media was completely devoid of ^{14}C-citrate uptake activity in the presence of Ni^{2+} (not shown) and, similarly, wild-type cells grown in LB medium did not show any uptake of ^{14}C-citrate (Fig. 2a).

Toxicity of Me^{2+}-citrate in the CcpA-deficient strain CM010

B. subtilis strain CM010 is deficient in CcpA, a central component in carbon catabolite repression in *B. subtilis*. Growth of the CM010 strain in LB medium resulted in significant uptake of ^{14}C-citrate in the presence of Ni^{2+} [initial uptake rate 0.2 nmol min^{-1} (mg protein)$^{-1}$)], indicative of functional expression of CitM (Fig. 2a). Apparently, expression of CitM in LB medium is repressed

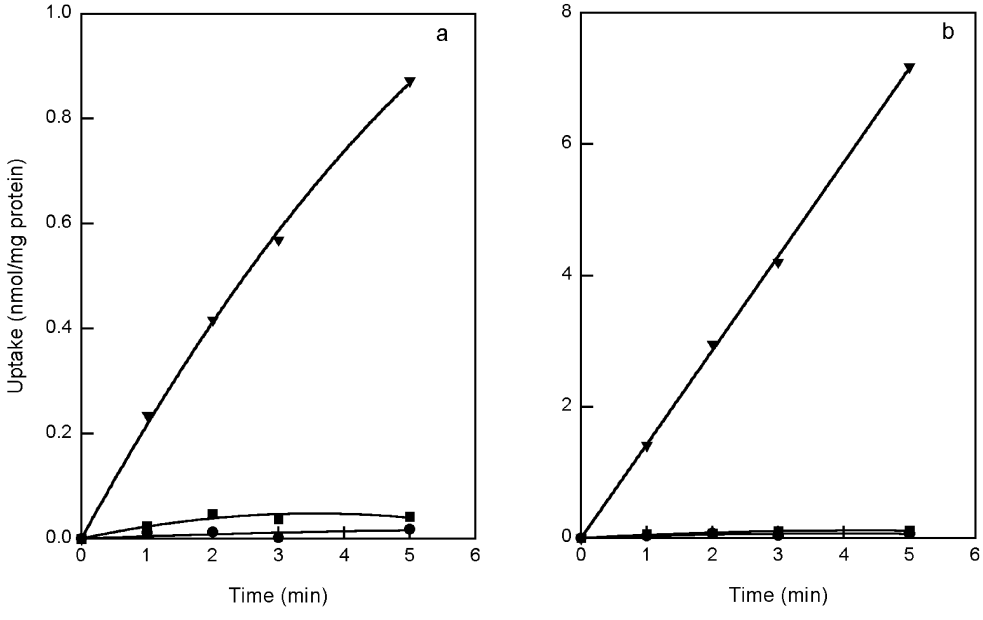

Fig. 2 Uptake of [1,5-^{14}C]citrate and $^{63}Ni^{2+}$ in resting cells. Uptake of [1,5-^{14}C]citrate in the presence of 1 mM $NiCl_2$ (**a**) and $^{63}Ni^{2+}$ in the presence of 100 µM citrate (**b**) in the wild-type strain (●), the CcpA-deficient strain CM010 (▼), and the NiR strain (■) grown in LB containing 10 mM citrate was measured in 50 mM PIPES buffer pH 6.5

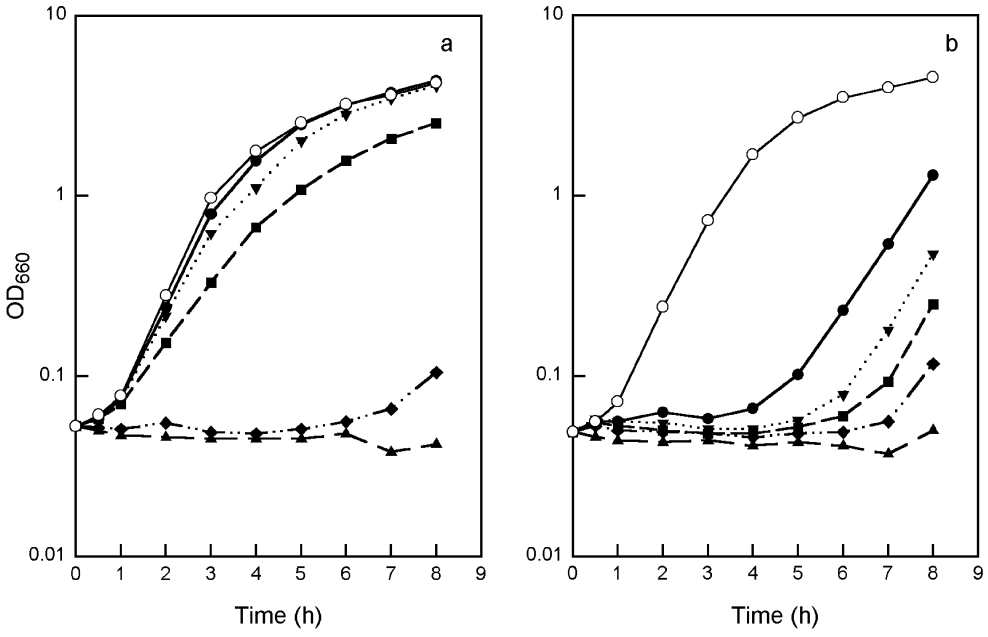

Fig. 3 Growth of the CcpA-deficient strain CM010 in LB (**a**) and LB containing 10 mM citrate (**b**) and concentrations of $ZnCl_2$ of 0.0 (○), 0.2 (●), 0.4 (▼), 0.6 (■), 0.8 (♦), and 1.0 (▲) mM. In the absence of added metal ions, the growth rates in the absence (**a**) and presence of citrate (**b**) were similar (1.3 h^{-1})

via the carbon catabolite repression system. To demonstrate that expression of CitM under these conditions resulted in enhanced uptake of toxic metal ions, uptake of $^{63}Ni^{2+}$ was measured. While the wild-type showed no uptake of $^{63}Ni^{2+}$ in the presence of excess citrate, the CcpA-deficient strain revealed significant $^{63}Ni^{2+}$ uptake activity with an initial uptake rate of 1.6 nmol min^{-1} (mg protein)$^{-1}$ (Fig. 2b). The difference in initial uptake rates of the Ni^{2+}-citrate complex when measured as ^{14}C-citrate uptake or $^{63}Ni^{2+}$ uptake is caused by the different complex concentrations in the two experiments (4.5 and 12 µM, respectively) which are below the affinity constant for uptake of the Ni^{2+}-citrate complex (K_m=43 µM; Krom et al. 2000). In addition, ^{14}C-citrate is rapidly metabolized in the cells and the label diffuses out of the cell as $^{14}CO_2$, while $^{63}Ni^{2+}$ is not metabolized.

In the absence of citrate, the toxicity of Zn^{2+}, Co^{2+}, and Ni^{2+} for the CcpA-deficient *B. subtilis* strain was similar to that observed for the wild-type strain (compare Figs. 3a and 1a, for Zn^{2+}). In contrast, in the presence of citrate, already low concentrations of the toxic ions had a dramatic effect on the growth behavior of the CcpA-deficient strain. For instance, 0.2 mM $ZnCl_2$ hardly effected growth in the absence of citrate, while in the presence of citrate the strain failed to grow for about 4 h after which growth resumed (Fig. 3). At higher concentrations of $ZnCl_2$ in the growth medium, the lag time increased. Similar observations were made for media containing Co^{2+} and Ni^{2+}. Clearly, during the first hours of incubation, the presence of citrate dramatically increased the toxicity of the metal ions in the case of the CcpA-deficient strain, while the opposite was observed for the *citM* deletion strain and the wild-type strain. The enhanced toxic effects of the heavy metal ions in the CcpA-deficient strain correlated with the presence of CitM activity and enhanced uptake of $^{63}Ni^{2+}$ in the cells (Fig. 2).

Development of Me^{2+} tolerance

The recovery of growth of the CcpA-deficient strain CM010 in the presence of the toxic Me^{2+}-citrate complexes after a prolonged lag time suggested the development of increased tolerance towards the Me^{2+}. Growth of the CM010 strain in the presence of citrate and 0.6 mM Zn^{2+} resulted in a lag phase of 6–7 h (Fig. 3). After 24 h of growth, the culture reached a similar cell density as a culture with no Zn^{2+} added. The overnight culture grown in the presence of 0.6 mM Zn^{2+} was used to inoculate LB containing 10 mM citrate in the absence and presence 0.6 mM Zn^{2+}. In the presence of Zn^{2+} the long lag phase as observed on day one was absent, and the growth rates in the two media were indistinguishable (not shown). Clearly, the culture had adapted to the presence of Zn^{2+} in the medium by developing a higher tolerance towards Zn^{2+}. The same phenomenon was observed for Co^{2+} and Ni^{2+} when present at toxic levels in the growth medium.

Cells taken from cultures adapted to toxic levels of 0.6 mM Zn^{2+}, 0.4 mM Co^{2+}, or 1 mM Ni^{2+} were plated on LB plates yielding single colonies (metal-tolerant strains ZnR, CoR, and NiR, respectively). A single colony of each of the tolerant strains was used to inoculate fresh medium and the culture was grown for several generations in the absence of the metal ions, after which the cells were grown under the original toxic conditions. All three strains were still tolerant, indicating that the phenotype was stable. The three metal-tolerant strains were tested for cross-tolerance against the other metal ions. Overnight cultures prepared from single colonies were diluted 1:100 in fresh LB containing 10 mM citrate and 0.6 mM $ZnCl_2$, 1 mM $NiCl_2$, or 0.4 mM $CoCl_2$. In all cases, the lag time observed for the CM010 strain was absent (not shown). In conclusion, a strain with increased tolerance towards one of the metal ions was also tolerant towards the two others.

Tolerance mechanism

In the presence of citrate, strain CM010 showed $^{63}Ni^{2+}$ uptake with an initial rate of 1.6 nmol min^{-1} (mg protein)$^{-1}$ (Fig. 2b). In contrast, none of the metal-tolerant strains showed any $^{63}Ni^{2+}$ uptake activity (see Fig. 2b for the NiR strain). Apparently, the metal-tolerant strains had lost their ability to accumulate the toxic metal ions. Similarly, in the presence of $NiCl_2$, CM010 took up ^{14}C-citrate with an initial rate of 0.2 nmol min^{-1} (mg protein)$^{-1}$, while the metal-tolerant strains did not take up ^{14}C-citrate under the same conditions (Fig. 2a). The lack of Ni^{2+}-dependent citrate uptake and citrate-dependent Ni^{2+} uptake in the metal-tolerant strains indicated the absence of active CitM in the cell membrane, which allowed the metal-tolerant strains to grow in higher concentrations of Me^{2+} in the presence of citrate than the parental CM010 strain.

The stability of the tolerance of the different strains suggested that the lack of CitM activity was due to the lack of synthesis of CitM in the cell, which may be due to mutations in the two-component sensory system CitST, in the promoter region, or in the *citM* gene. Strain CM010 and, therefore, the metal-tolerant strains, contain a fusion of the *citM* promoter region and the gene coding for β-galactosidase *lacZ* integrated in the chromosome at a location distant from the *citM* gene (P*citM-lacZ* promoter fusion; Warner et al. 2000). The promoter fusion was used to test the integrity of the two-component system. β-Galactosidase activity of CM010 cells grown in LB medium in the absence of citrate was very low, while growth in the presence of citrate resulted in β-galactosidase activities of 26 Miller units (Table 1). The metal-tolerant strains revealed β-galactosidase activities in the presence of citrate similar as observed for the parental CM010 strain, indicating a functional CitST two-component system.

Subsequently, the *citM* promoter region in front of the *citM* gene as well as the structural gene of *citM* were amplified from two independent metal-tolerant strains using PCR and the nucleotide sequence was determined. The promoter region of *citM* contains the CitT binding site (Yamamoto et al. 2000), the transcription start point, the catabolite responsive element (CRE-site) (Warner et al. 2000) and the ribosome binding site – all elements involved in the transcription of *citM*. Sequencing of the promoter region of the two metal-tolerant strains revealed no mutations (not shown). Sequencing of the *citM* gene revealed that in both cases a single point mutation had occurred. One strain contained a C to A mutation in the TAC codon for Phe at position 349 resulting in a TAA stop codon at that position, which would result in an incomplete CitM molecule missing about 20% of the residues at the C-terminus. The other strain contained a G to T mutation in the GAA codon for Glu at position 199, also resulting in a stop codon and truncating CitM by more than 50%.

Table 1 Promoter activities of *citM* in metal-tolerant strains. β-Galactosidase activity of *Bacillus subtilis* strains containing a P*citM-LacZ* promoter fusion was measured after 7 h of growth in LB medium in the presence or absence of 10 mM citrate

Strain	Citrate in the medium	LacZ activity (MU)
CM010	–	0.3
	+	26
ZnR	+	21
CoR	+	21
NiR	+	20

Discussion

One approach in the bioremediation of heavy-metal-polluted sites and waste water is the use of chelating agents (Peters 1999). The use of citrate as the chelator may be particularly interesting, since citrate, on the one hand, is produced in situ by specific microorganisms (e.g. White et al. 1997) while, on the other hand, some microorganisms are capable of accumulating heavy metals complexed to citrate (Madsen and Alexander 1985). Combination of the two processes would result in a complete cycle of bioremediation in which the toxic metal, in the first step, is mobilized from the contaminated site by binding to citrate, and, in the second step, is recovered from the leachate by bioaccumulation. *B. subtilis* expressing the Mg^{2+}-citrate transporter CitM could potentially be useful in the latter step as the transporter accepts the toxic heavy metal ions Zn^{2+}, Co^{2+}, and Ni^{2+} instead of Mg^{2+} in the complex with citrate (Krom et al. 2000). A potential drawback of the bioaccumulation of heavy metal ions may be increased toxicity at relatively low concentration.

Most microorganisms are protected against the toxic effects of heavy metal ions by the addition of citrate to the growth medium. The metal ions are taken up by transporters that are specific for the free metal ions and do not recognize the Me-citrate complex. Also, while most microorganisms have transporters for citrate, these transporters do not recognize the Me-citrate complex and transport only the free citrate anion. A simple application of this is the use of citrate in microbial growth media to complex metals, thereby reducing their toxicity (Brynhildsen and Rosswall 1989). The protective effect of citrate is demonstrated here for *B. subtilis* when CitM is not present in the membrane (Fig. 1). Clearly, protection by citrate is compromised when transporters are present in the membrane that transport the complex of the toxic metal ions and citrate. Then, the new entrance pathway for the metal ions into the cell may even increase toxicity. It is demonstrated that the dramatically increased sensitivity of *B. subtilis* to Zn^{2+}, Co^{2+}, and Ni^{2+} ions in the presence of citrate under conditions when CitM was expressed (Fig. 3) correlated with enhanced Ni^{2+}-citrate transport into the cell (Fig. 2). Toxicity sets an upper limit to the concentration of the toxic metal ions in the bioaccumulation process. Under the conditions of the experiments presented in Fig. 3, more or less normal growth of *B. subtilis* in the presence of concentrations of Zn^{2+}, Co^{2+}, and Ni^{2+} of 100, 20, and 200 μM, respectively, was observed.

Toxic concentrations of the metal ions initially completely inhibited growth of *B. subtilis*; however, after some time the cultures adapted to the presence of the

metal ions and, subsequently, showed the original growth behavior. The development of resistance against heavy metal ions is a generally observed phenomenon. Most commonly, the mechanism of resistance in prokaryotes is efflux of the toxic metals by the action of P-type ATPases or secondary efflux systems (Nies and Silver 1995; Paulsen and Saier, Jr. 1997). The genome of *B. subtilis* contains several ORFs with similarity to P-type ATPases and ORFs for cation efflux systems belonging to the CDF family of secondary transporters. Other less ubiquitous systems for metal resistance involve the expression of metal binding proteins like metallothioneins and, specific for mercury ions, chemical reduction to the metallic state (reviewed in (Silver 1996)). In this study, cultures became tolerant towards higher concentrations of Me-citrate. Analysis of the metal-tolerant strains of *B. subtilis* generated in this study showed that the cultures had adapted to the toxic conditions by inactivation of the uptake system. The cells contained point mutations in the structural gene coding for CitM that introduced stop codons, resulting in premature termination of translation and, consequently, truncated CitM molecules. The mechanism by which these point mutations may occur could be either spontaneous or mediated by a specific mechanism induced by the presence of the toxic ions. In the former mechanism, the adaptation process would involve selection of those cells with random mutations in the *citM* gene that inactivate the transporter. The observation that the time span after which growth occurred was reproducible and dependent on the metal ion concentration (Fig. 3) suggests involvement of a more specific mechanism for the introduction of the mutations. It is known that heavy metals are mutagenic by replacing essential metals like Mg^{2+} or Ca^{2+} in biomolecules (Lombardi and Garcia 1999) and thus influencing the function of these molecules. More research is required to resolve this issue. Nonetheless, the development of tolerance and resistance has the advantage that the cells can cope with higher concentrations of the metal ions in the medium. In terms of bioaccumulation, tolerance or resistance is only advantageous when the cells inactivate the ions by binding to macromolecules which effectively increases the total concentration of the toxic ions in the cells. Pumping out the metal ions or preventing influx is unwanted for this purpose.

The results presented here show that the Mg^{2+}-citrate transporter CitM promotes toxic metal ion accumulation in *B. subtilis* and increases the toxicity of these metal ions. The system could potentially have application as part of a bioremediation system of sites polluted with heavy metals involving citrate chelation followed by accumulation of the Me^{2+}-citrate complex by *B. subtilis*. However, metal ion concentrations should be low enough to prevent inactivation of the transporter. Overexpression of metal ion binding proteins like pea or yeast metallothioneins has been demonstrated to increase the resistance of *E. coli* against Ni^{2+} and Hg^{2+} ions (Chen and Wilson 1997; Krishnaswamy and Wilson 2000). Expression of these metal-binding proteins in *B. subtilis* may enhance the resistance to heavy metals without compromising the potential application. Alternatively, heterologous expression of CitM in a more metal-resistant host may be useful to enhance accumulation of the metal ions in the biomass.

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